Thin Films of Collagen Affect Smooth Muscle Cell Morphology†

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Received July 10, 2002. In Final Form: September 17, 2002

Thin films of the extracellular matrix protein, collagen, were prepared by adsorbing native or heat-denatured type I collagen onto hexadecanethiol self-assembled monolayers. The resulting films were characterized by atomic force microscopy, ellipsometry, and light microscopy. Denatured collagen formed a topographically smooth ∼3.6 nm thick film, consistent with an adsorbed protein monolayer. In contrast, the native collagen thin film consisted of supramolecular collagen fibrils. The density of the large fibrils could be varied by changing the native collagen concentration in the solution from which the films were prepared. The biomimetic nature of the thin collagen films was partially assessed by examining their effects on vascular smooth muscle cells. Automated quantitative analysis indicated that the morphology of smooth muscle cells on the thin films was dependent on whether the collagen was heat-denatured or was in its native fibrillar form. The area of cells on denatured collagen films was significantly larger than that of cells on thin films of native fibrillar collagen. This response closely mimicked the response of these cells to thick collagen gels. Examination of the relationship between collagen fibril density and cell area indicated that large fibrils play a role in determining how cells respond to collagen. Cells assumed a larger morphology on native collagen films with a lower density of large fibrils. In this study, it is clear that cell morphology on these films is determined by micron-scale interactions between cells and the matrix molecules and is not dependent on the bulk materials properties of collagen gels.

Introduction

The use of thin films of extracellular matrix (ECM) proteins or related peptides prepared by employing self-assembled monolayers or "soft lithography" is of increasing interest in studies of cell−surface interactions.[1−4] These approaches provide the means for achieving highly reproducible biomolecular assemblies on planar surfaces that can be analyzed with precision and verified to be similar from preparation to preparation. The molecular composition of thin films can be systematically altered, molecular components can be organized into precise two-dimensional spatial arrangements, and the topography of surface features can be controlled. By providing a means to alter these parameters independently, thin films make it possible to examine the effect of such features on cell response. These attributes make thin films applicable to engineering and are often not achievable by using polymers or hydrogels or by adsorbing ECM proteins onto inhomogeneous glass or plastic surfaces. Thin film techniques have been used to control ECM ligand density in order to study the strength of cell binding[5] and the spatial arrangement of ECM proteins in order to study the relationship between cytoskeletal structure and apoptosis.[6]

The ECM controls many aspects of cell behavior, including growth, differentiation, migration, and survival.[7,8] A major ECM protein component of most mammalian tissues is type I collagen. It is synthesized in cells as hydroxylated subunits that form long, triple helical domains (∼1.5 nm wide, 300 nm long[9]); these polypeptide complexes are subsequently transported out of the cell, where they assemble into large, supramolecular fibrils which then interact with one another in more extensive associations. The collagen network plays both a structural and a signaling role in the physiological response of smooth muscle cells (SMCs) within adult muscularized arteries. In healthy tissue, type I collagen provides signals that direct SMCs to remain in a mostly nonproliferative state. However, following vascular injury, cell-associated ECM-degrading proteases modify type I collagen in a way that promotes SMC proliferation,[10−14] possibly by making cryptic binding sites available to cell surface receptors.[15]

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† Part of the *Langmuir* special issue entitled The Biomolecular Interface.

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10.1021/la026216r CCC: $25.00 © 2003 American Chemical Society
Published on Web 10/23/2002

To model the normal in vivo condition of vascular SMCs in a collagen matrix,[12] hydrogels of collagen fibrils are formed from solutions of native, monomeric triple helical type I collagen under appropriate conditions of pH, ionic strength, concentration, and temperature.[16] The post-injury state is modeled by culturing SMCs on more rigid, dried gels of heat-denatured collagen. SMC responses to these model gel matrixes are similar to their responses in vivo: the thick gels of native fibrillar collagen do not support SMC proliferation,[12,17] and gels of heat-denatured collagen promote cellular proliferation.[12] While it is well established that denatured type I collagen and native type I collagen interact with different cell surface receptors to evoke different responses from cells,[11,13,15,18] data also suggest that SMCs maintained on native fibrillar collagen have a distinct morphology and appear to be smaller than cells grown on denatured collagen.[12−14,17] A number of studies strongly suggest that particular cellular morphologies are closely linked to, and may be required for, activation of cellular proliferation and differentiation.[6,7,19−21] However, the precise relationship between signals that direct cell morphology and signals that direct cell proliferation remains unresolved. Furthermore, heat-denaturation of collagen, like the action of ECM-degrading proteases, destroys the ordered three-dimensional, triple helical structure of native type I collagen, leaving random coils that are unable to assemble fibrils.[22] The mechanism by which mechanical differences in native and denatured collagen matrixes influence cell morphology and proliferation is not fully understood.

As a model system for such studies, thick collagen gels have the disadvantage of being difficult to prepare consistently from experiment to experiment. The native collagen gels can easily become partially detached from their supporting surfaces, and macroscopic physical differences in the gel can frequently be seen by eye. The properties of the thick gels can be difficult to analyze quantitatively to ensure reproducibility of the preparations. In addition, quantitative analysis of cell characteristics on such matrixes can be difficult due to light scattering from the fibrillar gel and trapping of fluorescent reagents. Collagen monomers immobilized on polystyrene, polycarbonate, or acrylamide gels have been used as alternative model systems,[17,23,24] but the surface properties of such substrates have not been fully characterized.

We describe here the preparation and characterization of thin collagen films and the assessment of their biomimetic nature by comparing the morphology of SMCs on the thin films to the morphology of SMCs on thick collagen gels. The use of thin films of fibrillar collagen offers the advantages of excellent visualization of intracellular structures and cellular interaction with the collagen matrix and of assessment of the reproducibility of the matrix preparation. By systematically altering the density of collagen fibrils in the thin films, these studies have allowed us to begin to understand the contribution of the collagen fibrils in determining shape and proliferative responses of SMCs.

Materials and Methods[25]

Preparation of Alkanethiol-Coated Supports. Glass coverslips (no. 1, 22 mm × 22 mm) were washed in 1% (w/v) sodium dodecyl sulfate/water, rinsed extensively with deionized H_2O (Millipore Corp, Bedford, MA), and acid-washed in fresh H_2SO_4 containing 10% potassium persulfate (30 min). The coverslips were rinsed extensively with deionized H_2O, transferred to acetone, and dried on a particle-free polyester cloth (Texwipe TX1010, Fisher Scientific, Springfield, NJ). Polished Si wafers (Silicon, Inc., Boise, ID) were cleaned by wiping them across an ethanol-soaked Kimwipe (Kimberly-Clark, Roswell, GA) and then a dry Kimwipe. Residual fiber and dust particles were removed under a stream of particle-filtered N_2. Coverslips and wafers were coated with a layer of chromium (5 nm) and a layer of gold (15−20 nm) by magnetron sputtering.[26] The gold-coated wafers and coverslips were immersed in 1-hexadecanethiol (0.5 mM; Aldrich, Milwaukee, WI) in ethanol for at least 8 h before being rinsed with ethanol and dried with filtered N_2. Alkanethiol-coated samples could be stored under ethanol for at least 7 days without any loss in performance.

Preparation of Thick Collagen Gels. Purified type I collagen was purchased as a solution of acid-stabilized monomer (Vitrogen; Cohesion Technologies, Inc., Palo Alto, CA). Heat-denatured collagen was prepared by mixing Vitrogen (∼3 mg/mL), 0.4 M acetic acid, and 10× Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate buffered saline (DPBS) in a 16:1:2 ratio, boiling the solution for 30 min, and neutralizing it with 0.5 M NaOH.[13] Neutralized denatured collagen solution (350 μL) was placed into the center of an ethanol-sterilized silicone gasket (20 mm diam; Electron Microscopy Sciences, Fort Washington, PA) that was affixed to a cleaned, ethanol-sterilized glass coverslip. Samples were air-dried overnight in a sterile hood, rinsed several times with DPBS, and stored in DPBS at 4 °C until they were used. The silicone gaskets were removed before the cell culture experiments were initiated.

To prepare thick gels of native fibrillar collagen, a variation of a published method[13] was used. Gaskets (∼16 mm inside diam, 22 mm outside diam) were cut from paper labels (Fasson label material; Avery Dennison, Brea, CA) and adhered onto cleaned coverslips before sterilization with 70% ethanol. The paper gaskets aided in preventing the collagen gel from sliding off the glass coverslip. Vitrogen collagen solution (0.8 mL, ∼3 mg/mL, 4 °C) was neutralized with 0.1 mL of 10× DPBS (4 °C) and 0.1 mL of NaOH (0.1 M, 4 °C) and was kept at 4 °C to minimize polymerization. Neutralized native collagen solution (100 μL) was applied to the dried coverslips, making sure that solution contacted the inside edge of the paper gaskets. The samples were placed at 37 °C overnight to initiate fibrillogenesis. The native collagen gels were carefully rinsed and stored in DPBS at 4 °C until they were used.

Preparation and Characterization of Thin Collagen Films. Thin films of denatured collagen were prepared by immersing alkanethiol-treated gold-coated glass coverslips or Si wafer pieces (∼2 cm^2) into a solution of denatured collagen in DPBS (∼0.3 mg/mL final concentration). The samples were incubated at 4 °C for at least 12 h, rinsed in DPBS and water, and stored in DPBS at 4 °C until ready for use with cells. For all samples used in this study, the denatured collagen thin films were not allowed to dewet during this rinsing procedure.

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Subsequent data indicate that dewetting, drying, and rehydrating the thin films of denatured collagen does not influence their characteristics as determined by SMC morphology.

To prepare thin films of native collagen, alkanethiol-treated gold-coated coverslips or Si wafer pieces were placed into neutralized solutions of native collagen in DPBS (4 °C). The immersed samples were then incubated overnight at 37 °C. After incubation, the samples were slowly lifted out of the gelled collagen solutions and rinsed by immersing into DPBS (∼10 times) and deionized water (∼10 times) to remove all loosely adhered gel. The samples were then dried under a stream of filtered N_2 and immediately placed back into a DPBS solution. The samples were stored in DPBS at 4 °C until they were used.

The thickness of the collagen thin films on Si substrates was determined by spectroscopic ellipsometry (J. A. Woollam, model M-44, Lincoln, NE) using a two-layer model. The optical constants of the first layer were determined empirically from ellipsometric data collected for a control sample of alkanethiol-treated, gold-coated Si wafer. The optical constants of the second layer were fixed to $n = 1.45$ and $k = 0$ to approximate the properties of the protein film, and the average thickness of the second layer for each sample was determined using the manufacturer's fitting routine.

Atomic force microscopy (PicoScan; Molecular Imaging, Phoenix, AZ) of the collagen thin films on Si substrates was performed in air using Si tips in magnetically driven, intermittent contact mode. Images were taken from several areas on each sample to ensure the homogeneity of surface features. Images were flattened and, in some cases, plane-fitted with the PicoScan software to improve visualization and analysis of the images. Image analysis was also performed with the PicoScan software. Imaging was also performed on some samples under DPBS in a fluid cell. The results indicated that hydrated and dry samples were qualitatively the same.

Texas Red Labeling of Collagens. To assist in characterization of the thin films, some films were prepared from fluorophore-labeled collagen, which facilitated their observation by fluorescence microscopy. Texas Red labeled denatured collagen was prepared by adding Texas Red sulfonyl chloride (0.5 mg; Molecular Probes, Eugene, OR) in 30 μL of dimethyl sulfoxide (DMSO) to a neutralized denatured collagen solution (1.6 mL, 2.4 mg/mL) prepared as described above. After 30 min at 4 °C, the sample was placed in 10 000 MW cutoff dialysis bags (Fisher Scientific) and dialyzed extensively in DPBS at 4 °C. Texas Red labeled native collagen was prepared by adding Texas Red sulfonyl chloride (0.5 mg) in 30 μL of DMSO to a neutralized native collagen solution (1.6 mL, 2.7 mg/mL, 4 °C) prepared as described above. After 30 min at 4 °C, the sample was acidified with concentrated HCl (16 μL), placed in 10 000 MW cutoff dialysis bags, and extensively dialyzed in 0.012 M HCl at 4 °C. The final concentrations for both labeled protein solutions were determined by the modified Peterson assay[27] using Vitrogen collagen solution as a protein standard. The Texas Red labeled native collagen retained its ability to form collagen fibrils at 37 °C that appeared by light microscopy to be identical to those formed from nonlabeled native collagen.

Cell Culture and Preparation for Microscopy. The smooth muscle cell line, A10 (American Type Culture Corp., Manassas, VA), was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with amino acids and 10% fetal bovine serum (FBS; Gibco Invitrogen, Carlsbad, CA). Prior to seeding cells on the collagen substrates, the thin films and thick gels were conditioned for a minimum of 3 h with three changes of DMEM (Mediatech, Herndon, VA) containing 2% FBS. This step was necessary to exchange solution trapped in gels with culture medium and to normalize the gel and film substrates with respect to possible adsorption of serum proteins. Cells were removed from tissue culture polystyrene flasks by trypsinization, washed with medium containing 2% FBS, plated onto the collagen substrates at a density of 3500−4500 cells/cm^2 in DMEM containing 2% FBS, and incubated at 37 °C for 24 or 48 h. The reduced serum concentration maximizes the extent of cell signaling that is due to the ECM and mimics conditions that have been typically used in characterizing the response of these cells to native and denatured collagen gels.[12] After incubation at 37 °C, cells on the coverslips were washed with warm Hanks balanced salt solution (HBSS; ICN Biomedicals, Costa Mesa, CA), fixed in 4% formaldehyde in DPBS (30 min at room temperature), quenched in 0.25% NH_4Cl in DPBS (15 min), permeabilized with 0.1% Triton X-100 in DPBS (5 min), rinsed three times in DPBS and once in DPBS−glycerol (1:1), and incubated with Texas Red−C_2−maleimide (Molecular Probes) in DPBS−glycerol (3.3 μg/mL, 45 min at room temperature). After extensive washing in DPBS−glycerol, substrates were mounted upside down on slides in Tris buffered saline (10 mM Tris, 140 mM NaCl, pH 8.5) containing 80% glycerol, 0.25% DABCO (Sigma, St. Louis, MO) to reduce photobleaching, and DAPI (1.5 μg/mL, Sigma) as a nuclear counterstain. Coverslips were sealed at the edges with nail polish. Throughout the fixing and staining procedure, cell samples were always kept immersed in solution. This avoided disruption of the cell structure by dewetting of the substrate, to which the denatured collagen samples were particularly susceptible.

Table 1. Surface Characterization of Collagen Thin Films

absorbed collagen	film thickness (nm)[a]	rms roughness (nm)[b]
denatured (4 °C, 330 μg/mL)	3.6 ± 0.5	0.8[c]
native[d] (37 °C, 25 μg/mL)	6.8 ± 0.5	3.4 ± 2.1
native[d] (37 °C, 75 μg/mL)	14.1 ± 1.3	13.8 ± 0.5
native[d] (37 °C, 250 μg/mL)	24.1 ± 2.0	16.5 ± 1.2
native[d] (37 °C, 750 μg/mL)	37.8 ± 2.0	21.6 ± 1.4

[a] Determined by spectroscopic ellipsometry, $n > 2$ samples. [b] Determined from AFM images over a 25 μm^2 area. [c] The rms roughness of a control hexadecanethiol-treated gold-coated Si wafer was ∼0.8 nm. [d] Prepared under conditions that initiated fibril formation.

Morphology Analysis. The fixed and stained cells were examined by phase contrast and fluorescence microscopy using an inverted microscope (Zeiss Axiovert S100TV, Thornwood, NJ) outfitted with a computer-controlled stage (LEP, Hawthorne, NY), an excitation filter wheel (LEP), and a CCD camera (CoolSnap fx, Roper Scientific Photometrics, Tucson, AZ). Hardware operation and image digitization and analysis were under software control (Inovision Inc., now ISEE Imaging, Cary, NC). A modular software routine controlled automated movement of the stage, autofocusing, and collection of data from 50 fields of cells per coverslip (using a 10× objective). At each field, cellular fluorescence from Texas Red, and then DAPI, was collected by automated switching of the appropriate excitation filters and passing the emitted light through a multipass beam splitter (set no. 84000; Chroma Technology Inc., Brattleboro, VT). For quantitative analysis, appropriate thresholding criteria allowed cell areas (as determined by cellular Texas Red fluorescence) to be accurately distinguished from the nonfluorescent noncell areas. The number of nuclei, and therefore the number of cells, was determined from the corresponding images collected with the DAPI filter. The requirement for spatial correspondence of DAPI and Texas Red fluorescence prevented noncellular fluorescent area from being included in the data set. Every Texas Red fluorescent area that was associated with DAPI staining was identified as a cell object. The average area per cell was calculated by dividing the area corresponding to Texas Red fluorescence by the number of nuclei as determined by DAPI staining. Each histogram and average area reported reflect the combined data from ∼500 cells.

Results

Fabrication and Characterization of Thin Films of Type I Collagen. Adsorption of denatured collagen in solution to the hexadecanethiol surface resulted in a protein film that was determined by ellipsometry to be 3.6 ± 0.5 nm thick (Table 1), consistent with the thickness of a collagen protein monolayer. Ellipsometric evaluation of ∼7 mm^2 sampling areas over a ∼1 cm^2 area indicated that the film thickness varied less than 7% across the surface. When films were formed from Texas Red labeled

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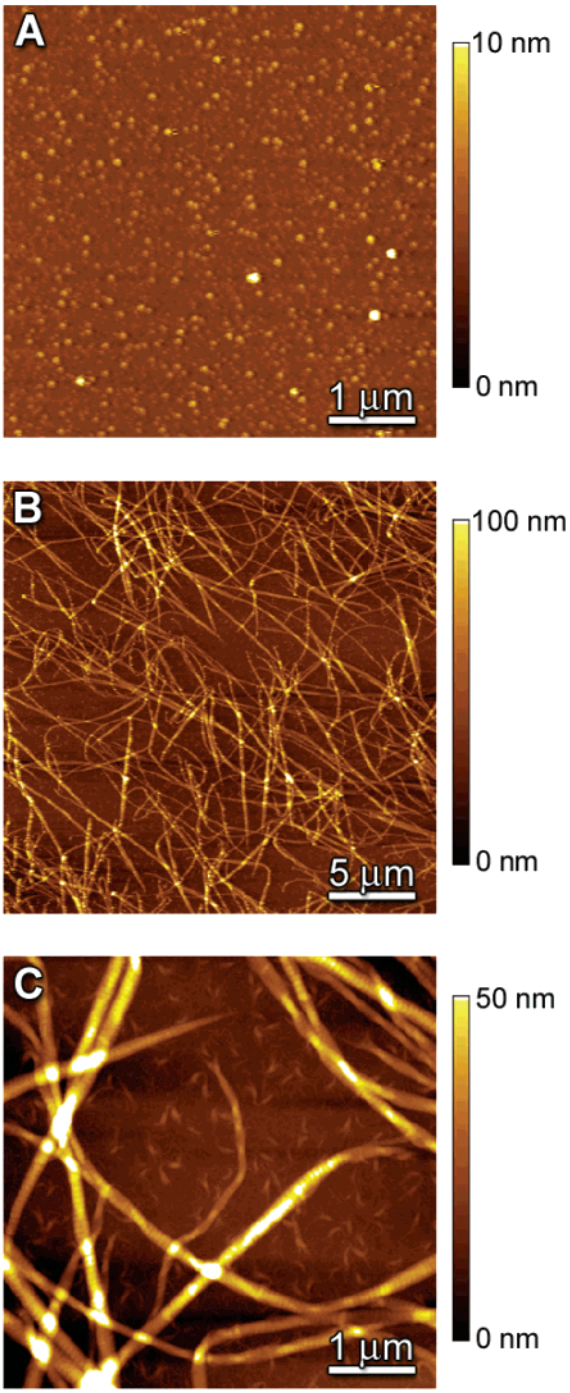

Figure 1. AFM images of thin films of type I collagen formed from solutions of ~330 μg/mL collagen. All surfaces were imaged in air. (A) Denatured collagen thin film, 5 μm × 5 μm scan area, full *Z*-scale = 10 nm. (B) Native collagen thin film, 25 μm × 25 μm scan area, full *Z*-scale = 100 nm. (C) Native collagen thin film, 5 μm × 5 μm scan area, full *Z*-scale = 50 nm.

denatured collagen, fluorescence microscopy indicated homogeneous surface coverage on a scale of microns to millimeters. Atomic force microscopy (AFM) images revealed a relatively smooth surface with a root-mean-square (rms) roughness of approximately 0.8 nm over a 25 μm^2 area (Figure 1A). This roughness value is on the order of the underlying Au film and indicates that large protein aggregates are not present at the denatured collagen surface.

In comparison, examination of hexadecanethiol surfaces incubated in solutions of neutralized native collagen (330 μg/mL) at 37 °C revealed that collagen fibrils were present at the surface. The larger fibrils can be seen easily by light microscopy, and AFM images of the films in air showed a mixture of large fibrils ~200−250 nm in diameter (Figure 1B) and smaller fibrils between 50 and 100 nm in diameter (Figure 1C). The ellipsometric data indicated an effective film thickness of several tens of nanometers (Table 1), although clearly much of the surface is composed of large collagen fibrils that are dispersed at the surface. The measured thickness of replicate samples provided a standard deviation of film thickness of less than 10%. The homogeneity of surface coverage, evaluated by comparing ellipsometric determinations at several areas on each sample, indicated that the standard deviation in thickness across the samples was less than 4%. Phase microscopy using a 10× objective and fluorescence microscopy of films formed with Texas Red labeled collagen showed that the fibril density was relatively homogeneous over an area of ~1 cm^2.

As shown in Table 1, the effective thickness of the fibrillar film was dependent upon the initial concentration of the native collagen solution used for formation of the thin film. AFM images shown in Figure 2 indicate that the density of large fibrils is a function of the collagen concentration in the adsorbing solution (Figure 2A−D). At higher collagen concentrations (75−750 μg/mL), the surfaces are covered with large fibrils (~200−250 nm in diameter and at least several microns in length) that are similar in size to the fibrils most easily observed in native collagen gels. These fibrils appear to lie on a bed of intermediate size fibrils (~75 nm in diameter and ~250 nm in length) that can be seen in a 2 μm × 2 μm scan area (Figure 2F). These intermediate and large fibrils appear to lie on a bed of very small fibrils (<50 nm diameter and ~250 nm in length) that are most easily discerned in the sample prepared from the lowest concentration collagen solution and imaged in a 2 μm × 2 μm scan area (Figure 2E). In some images (Figure 3A,B), it is possible to discern that some fibrils appear to arise out of smaller fibrils below them. This observation suggests that perhaps the larger fibrils are physically connected to smaller fibrils which are in closest proximity to the surface of the support.

Qualitative Response of SMCs to Collagen Films. To assess the biological relevance of the collagen thin films, we compared the response of SMCs grown on thin films to that of SMCs grown on thick gels of collagen. On thick gels of denatured collagen, SMCs are well spread (Figure 4A). In contrast, on thick gels prepared from native type I collagen, SMCs appear smaller and assume a morphology that is characterized by numerous lamellipodia (Figure 4B). On thin films prepared from denatured and native type I collagen, a similar response is seen. SMCs on thin films of denatured collagen (Figure 4C) appear to be well spread and assume a morphology that is similar to that of the cells on the thick gels of denatured collagen. The morphology of SMCs on thin films of native collagen fibrils prepared from 750 μg/mL collagen solutions (Figure 4D) is very similar to that of cells on the thick gels of native collagen. The intimate relationship between the lamellipodia of the cells and the collagen fibrils can be seen more clearly on the native collagen thin film than on the corresponding thick gel. The cells appear to interact closely with the large collagen fibrils. Most cells are observed to be adhered adjacent to an area that is apparently devoid of large fibrils. An example of this is shown in Figure 4D (arrowhead). These clear areas are not present prior to cell seeding. We conjecture that these areas arise by the mechanical or chemical action of cells on the large fibrils. Collectively, these data suggest that the mechanical and

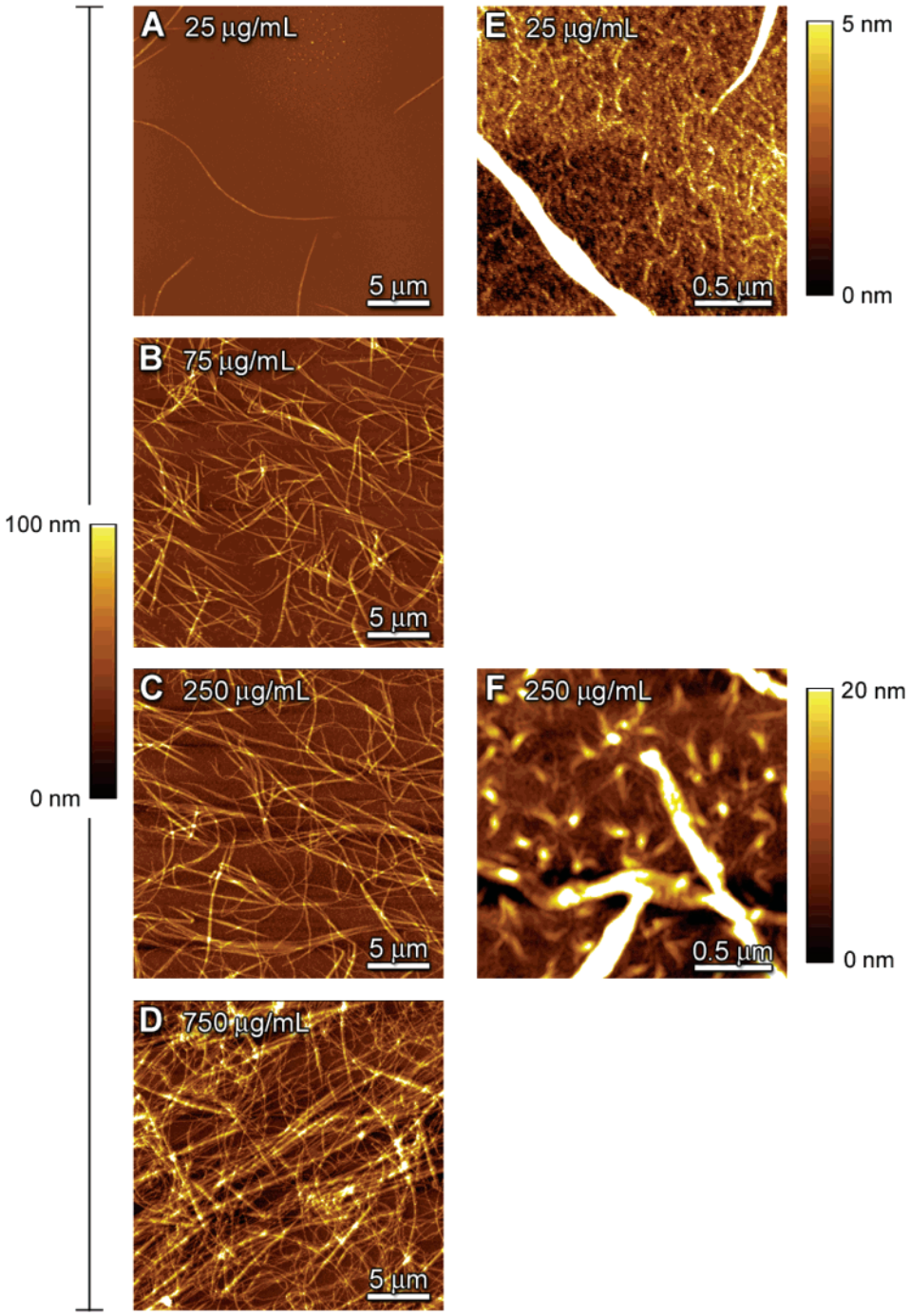

Figure 2. AFM images of native collagen thin films prepared from different concentrations of native collagen. (A−D) Scan size is 25 μm × 25 μm and full *Z*-scale = 100 nm. (E,F) Scan size is 2 μm × 2 μm and full *Z*-scale = 5 and 20 nm, respectively. The collagen solution concentrations are as shown.

chemical signals presented by the thin films of collagen mimic those presented by thick collagen gels.

Quantitative Analysis of SMC Area. Although cells on denatured collagen appear to be larger than those on native collagen, variations in cell sizes can be substantial, and a quantitative method was required to unequivocally determine and compare the sizes of cells on the different substrates. Quantitative determinations of cell size were achieved using fluorescence microscopy and image processing with Texas Red−C_2−maleimide stained cells (Elliott et al., manuscript in preparation). As shown in Figure 4E,F, this dye, which has a high quantum efficiency and a high selectivity for cellular components, provides excellent discrimination between the cell and noncell areas. Selection of fields, cell identification, and quantitative morphology analysis were totally automated, allowing unbiased data collection over many hundreds of cells. Representative histograms of cell sizes are shown in Figure 5. Figure 5A shows that SMCs on thick gels of denatured collagen are distinctly larger (mean cell area, ~4500 μm^2) than SMCs on thick gels of native type I collagen (mean cell area, ~1700 μm^2). In addition, it is clear that the width of the cell size distribution within the population on the native collagen gels is distinctly different than the width of the cell size distribution on the denatured collagen gels.

Data for cells grown on thin films of collagen are shown in Figure 5B,C. The mean size and distribution of sizes

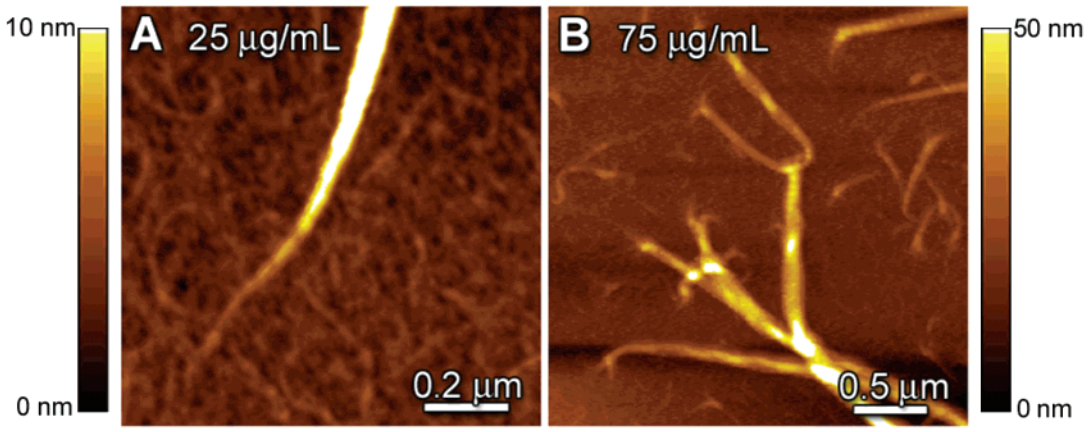

Figure 3. AFM images of native collagen thin films. The images show the ends of large fibrils that appear to be connected to smaller fibrils in close proximity to the surface. (A) Film prepared from 25 μg/mL collagen solution. Scan size is 1 μm × 1 μm and full *Z*-scale = 10 nm. (B) Film prepared from 75 μg/mL collagen solution. Scan size is 3 μm × 3 μm and full *Z*-scale = 50 nm.

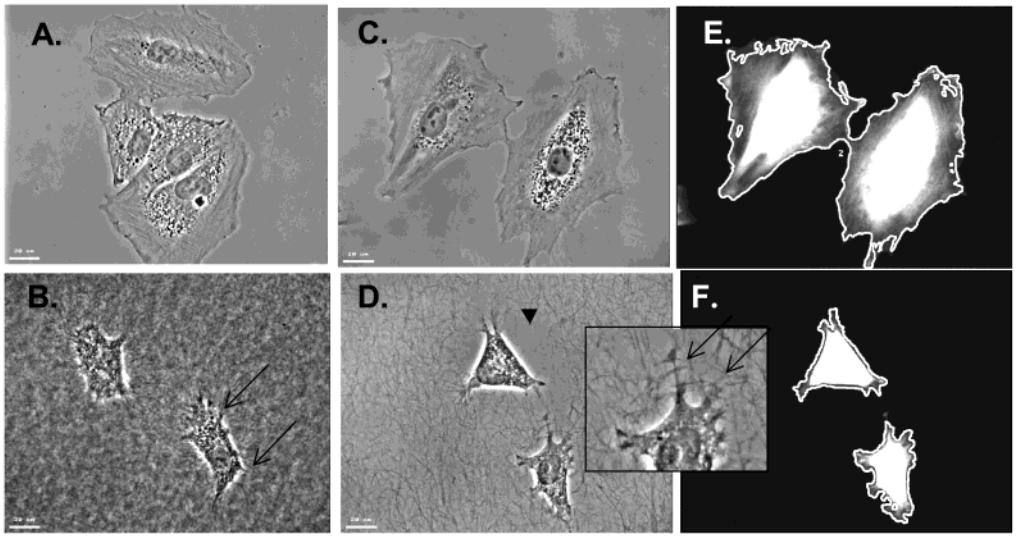

Figure 4. Optical microscopy of fixed SMCs on thick gels and thin films of type I collagen. All images were collected with a 40× objective. Phase contrast of SMCs on (A) a thick gel of denatured collagen, (B) a thick gel of native collagen, (C) a thin film of denatured collagen prepared from a solution of ~300 μg/mL denatured collagen, and (D) a thin film of native collagen prepared from a solution of 750 μg/mL collagen. The arrows in panel B and the inset of panel D indicate examples of lamellipodia in intimate contact with large collagen fibrils. The arrowhead in panel D indicates an area adjacent to a cell that appears to be cleared of the larger collagen fibrils. Fluorescence of Texas Red−C_2−maleimide stained SMCs on (E) a thin film of denatured collagen and (F) a thin film of native collagen. In panels E and F, Texas Red labeled cells were outlined based on discrimination of bright cell areas from dark background areas with the thresholding criteria that were used in the image analysis program during quantitation of the cell areas.

of SMCs on the thin films of denatured collagen are nearly identical to those of cells on the corresponding thick gels of denatured collagen (Figure 5B). The cells on the thin films of native fibrillar collagen prepared from 750 μg/mL collagen solutions are similar in size to the cells on thick gels of native collagen (Figure 5C). In addition, the cells grown on the native collagen thin films have a narrow size distribution similar to that seen for the cells grown on the thick gels of native collagen. Thus, SMCs assume different morphologies on native versus denatured collagen, regardless of whether the matrix is a thick gel or a thin film.

SMC Proliferation. We also detected differences in the number of cells present on thin films of denatured versus native collagen. After 24 h in culture, the density of cells on the thin films of denatured collagen was almost twice the density of cells on the thin films of native collagen prepared from 750 μg/mL collagen solutions. Identical results were observed for cells on the thick collagen gels. Although significant differences in cell adhesion cannot be excluded, this observation suggests that SMCs, which have been shown to be in a more proliferative state on denatured collagen gels as opposed to native collagen gels,[12] are also likely in a more proliferative state on the thin films of denatured collagen versus native collagen.

Varying Collagen Fibril Density. The results presented thus far indicate that thin films of collagen mimic thick gels with respect to their effects on SMCs. The thin films apparently present the appropriate chemical and mechanical features since the cell response on these surfaces is similar to the response on thick gel matrixes. The result is somewhat surprising for the thin films of native collagen since the bulk mechanical properties are very different from those of the thick native collagen hydrogels. We had, in fact, anticipated that the difference in morphology of cells on native versus denatured collagen gels may, in part, be due to the distinct differences in the mechanical properties of the thick native collagen hydrogel and the thick but more rigid gels of dried denatured collagen. However, since the morphology of SMCs on thin films of native collagen is quite similar to their morphology on thick gels of native collagen, we must conclude that

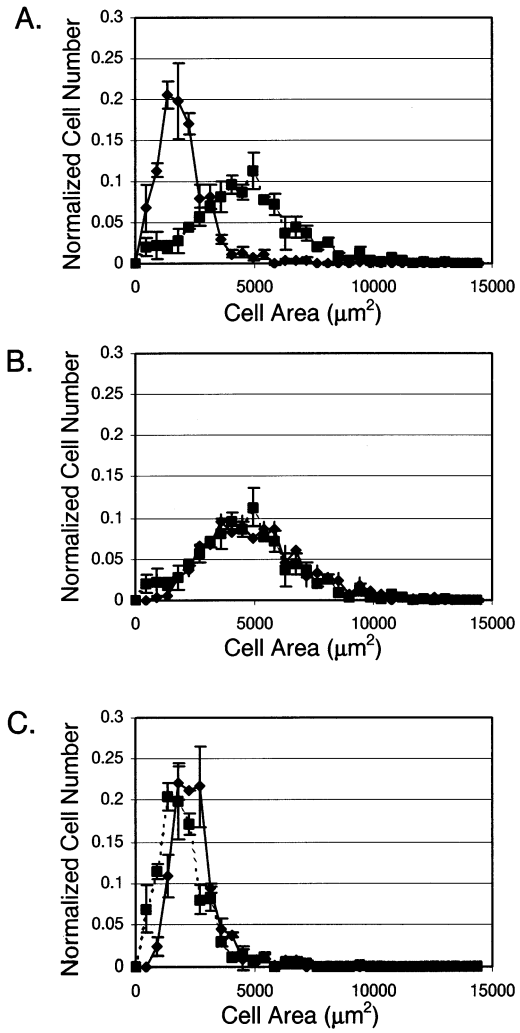

Figure 5. Representative histogram analyses of cell areas for SMCs on thick collagen gels and thin collagen films. (A) Cell areas on thick gels of native (◆, solid line) and denatured (■, dashed line) collagen. (B) Comparison of areas of cells on thick gels of denatured collagen (■, dashed line) and cells on thin films of denatured collagen (prepared from a solution of ~300 μg/mL) (◆, solid line). (C) Comparison of areas of cells on thick gels of native collagen (■, dashed line) and cells on thin films of native collagen (prepared from a solution of ~750 μg/mL) (◆, solid line). Error bars indicate the standard deviation from the results of at least two different experiments. Each histogram is based on data from ~500 cells.

this distinctive morphology is not directly determined by bulk properties of the matrix. Because the nature of the cellular interaction with the large fibrils in these two matrixes appears to be similar, we looked to the possible role of collagen fibrils as determinants of SMC response to native collagen.

The use of thin films allows us to study the role of fibrils in a systematic way. We found that the density of large fibrils can be tailored by changing the concentration of the collagen solution used in preparation of the thin films (see Table 1 and Figure 2). As the density of large fibrils decreased with decreasing collagen concentration, SMC spreading and average cell area increased (Figure 6). On thin films prepared from the solution of lowest concentration of native collagen (25 μg/mL), the average area of SMCs was similar to that of cells on denatured collagen substrates. On thin films prepared from high concentrations of collagen in solution (>250 μg/mL), cell size approached that of cells on native collagen hydrogels.

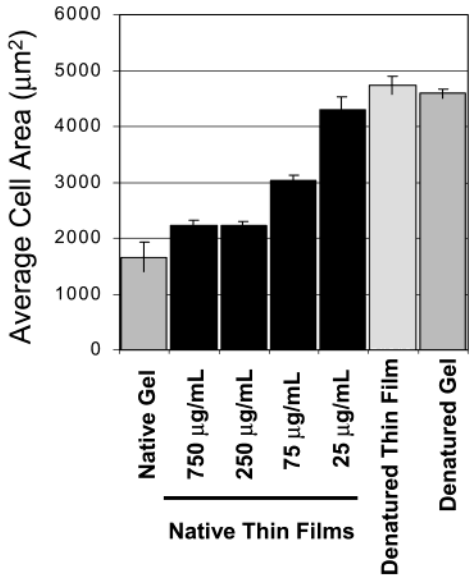

Figure 6. Effect of collagen fibril density on average SMC area. Thin films of native collagen (black bars) were prepared from solutions of different collagen concentrations under conditions in which collagen fibrils could form. For comparison, data are also shown for the mean area of cells on thin films of denatured collagen (white bar) and on thick gels of native and denatured collagen (gray bars). The ellipsometric thickness of the thin films is shown in Table 1.

These data may suggest that the presence of large collagen fibrils plays a significant role in determining the morphology and size of SMCs. The possibility that some of the collagen adsorbed from the low-concentration solutions becomes denatured at the surface, and that the large size is due to the cells being exposed to chemical signals associated with denatured collagen, must be considered. However, the AFM images of the sample prepared from the solution of 25 μg/mL collagen indicate the presence of tiny fibrils (Figure 2E), suggesting that the adsorbed collagen is in its native form because it is capable of polymerizing. Denis et al.[28] observed similar structures in collagen films prepared on alkanethiol monolayers from solutions containing low concentrations of native collagen and presented evidence for their manipulation under solution with the AFM tip. Therefore, we assume that the chemical properties of the adsorbed collagen prepared at this low concentration are those of native collagen. If this is the case, the difference in cell morphology may be due to mechanical differences between the large and small fibrils, and reducing the density of the large fibrils merely exposes the cells to the small fibrils. Further analysis will be required to confirm that the interaction of cells with all of the surfaces prepared with native collagen occurs through the same receptors. Nevertheless, the data taken as a whole strongly suggest that the small size of SMCs on native fibrillar collagen is dependent on the presence of large collagen fibrils.

Discussion

Advantages of Thin Films. Thin film ECM mimics provide new approaches for addressing biological questions regarding ECM−cell interactions and for applications such as live cell biosensors[1] and microfluidic systems that utilize

(28) Denis, F. A.; Hanarp, P.; Sutherland, D. S.; Gold, J.; Mustin, C.; Rouxhet, P. G.; Dufrene, Y. F. *Langmuir* **2002**, *18*, 819−828.

live cells.[29] The present work adds the ECM protein, collagen, to the list of thin film ECM mimics available for surface engineering in cell studies and applications. The methodology developed in this work will make it possible to engineer surfaces of type I collagen using photolithographic and/or soft lithography techniques.

The approach described here is based on the spontaneous adsorption of collagen from solution onto alkanethiol monolayers, which produces highly reproducible films. Previous work has shown that the ECM protein, fibronectin, also adsorbs readily to hydrophobic alkanethiol monolayers.[30] Denatured collagen, which has lost the triple helical structure essential for fibril formation, probably forms a monomolecular film of protein at the surface, based on ellipsometry and AFM data. Thin films prepared from native collagen at 37 °C exhibit supramolecular fibrils. AFM allowed us to identify the presence of three distinct populations of different size fibrils that form under conditions of different collagen concentrations. The three sizes of fibrils are similar to those that have been observed with electron microscopy of bulk collagen gel preparations at different time points in the polymerization process[16] and correspond to physiologically relevant stages in fibril formation and tissue maturation.[31] The exact nature of the physical connection between the small and large fibrils in the thin films is not clearly understood, but AFM images (Figure 3) suggest that the smallest fibrils lie closest to the support and possibly act as a seeding site for the growth of larger fibrils in the presence of increasing concentrations of collagen. The complex array of different size collagen fibrils that is apparent in these thin films may provide a variety of signals to the cells that interact with them. Thin films of ECM proteins provide the means of examining highly reproducible and systematically controllable matrixes with high-resolution techniques that can provide information that is important to fully understand, and eventually control, many of the signals presented to cells.

An additional advantage of thin films is that the rugged nature of these preparations allows samples to be stored for at least 1 week without any apparent damage to structural or functional integrity and allows the samples to be shipped to other laboratories.

The analysis of cells on thin films can be less ambiguous than on thick gels. While quantitative analysis of SMC area can be achieved for cells grown on thick collagen hydrogels (Chapados et al., submitted), it is significantly more challenging than making such measurements on thin films. Irregularities in the topography of thick gels cause changes in the focal plane, and scattered light from the thick matrix reduces the fluorescence signal-to-noise ratio. Furthermore, phase contrast microscopy of cells on thin films allows one to clearly see how cells interact with fibrillar collagen (Figure 4).

The Putative Role of Mechanical Features of Native Fibrillar Collagen. Thin collagen films allow us to control the supramolecular organization of collagen and examine its influence on cell response. We have demonstrated control over the density of large collagen fibrils present in the thin films. At the lowest concentration of collagen used (25 μg/mL), the surface appears to be covered with small fibrils (<50 nm in diam) and very few large fibrils (Figure 2A), while at higher concentrations of collagen examined, the density of large fibrils (~250 nm in diam) increased with increasing concentration of collagen (Figure 2B−D). This magnitude of change in large fibril density is not possible with bulk gels. A study to systematically alter the density of fibrils in the bulk gel was limited to a comparatively small range of collagen concentrations that could be as low as 0.3 mg/mL.[32] Altering fibril density significantly affected the tensile mechanical properties of the resulting bulk gels.

Using different collagen concentrations, we can produce thin films of native collagen that are covered by either very small fibrils or by large fibrils (Figure 2). We found that cells on thin films of very small fibrils do not exhibit the same morphology as cells on thin films with large fibrils and do not mimic cells on native type I collagen gels in vitro. Since all of the preparations are composed of native collagen that is presumably chemically identical, this suggests that other features are partially responsible for the observed effects on cell size. AFM data suggest that larger fibrils grow out of small fibrils that appear to be pinned to the surface. This might suggest that these different size fibrils may have different mechanical properties due to their differences in proximity and attachment to the surface. The larger fibrils may be expected to be more flexible. Phase contrast microscopy clearly demonstrates the intimate interaction between large fibrils and cells. These results might suggest that tensile properties of the individual collagen fibrils are important in determining SMC morphology. Bulk deformations in collagen gels are the result of cell interactions with individual fibrils, and possibly the tensile properties of these fibrils, not the bulk properties of the gel, are ultimately responsible for the observed SMC morphology. The data presented here suggest that the ability to measure the mechanical properties of individual collagen fibrils in these materials may provide unique insight into the parameters that determine mechanical control of cell response.

It is well established that mechanical forces play a critical role in cell differentiation and proliferation,[33] apoptosis,[6] motility,[23,34] ECM remodeling,[35] integrin receptor/ECM bond strength,[36] and other cellular responses. Mechanical forces can also determine cell shape,[20,23] which is thought to likely be an important determinant of cell response. The cells on the thin films of native collagen are very similar to but are not as small as the cells on native collagen gels (Figures 5C and 6). The mean area of cells on the native thin films (~2200 μm^2) is ~ 30% larger than that of the cells on the native gels. This is significantly smaller than the mean size of cells on denatured collagen gels or denatured collagen thin films (~4500 μm^2). Comparison of cell numbers on the different substrates suggests that proliferation rates on denatured collagen films and gels are similar and are significantly greater than that on native collagen films or gels. Therefore, these data suggest that thin fibrillar films may be good mimics of native collagen gels. Examination of the signaling pathways induced by these matrixes is ongoing. The details of how the physical parameters of collagen fibrils exert their effect on SMCs are yet to be determined.

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Conclusion

In this report, we have fabricated thin films of native and denatured type I collagen and have characterized these films by using surface analytical techniques and by quantifying their effects on the morphology and proliferation of vascular SMCs. By comparing cell morphology and cell number, we find that these thin films are good mimics of thick collagen gels. This work also indicates that SMC shape can apparently be determined by cell−collagen interaction on a micron scale, in the absence of a bulk collagen gel. Collectively, these data demonstrate that thin films of the extracellular matrix protein, collagen, can be fabricated in a manner that is amenable to use in engineering and for manipulating and studying cell behavior. Such reproducible and analytically tractable collagen matrix mimics may provide a unique tool for systematically examining how matrix organization and composition provide signals to cells.

LA026216R